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<b>(21) International Application Number:</b> PCT/US97/13657  <b>(22) International Filing Date:</b> 1 August 1997 (01.08.97)  <b>(30) Priority Data:</b> 60/023,220      2 August 1996 (02.08.96)      US  <b>(71) Applicant (for all designated States except US):</b> THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10550 North Torrey Pines Road, Mail Drop TPC-8, La Jolla, CA 92037 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SUTCLIFFE, J., Gregor [US/US]; 2253 Via Tiempo, Cardiff, CA 92007 (US). GAUTVIK, Kaare, M. [NO/US]; 3365 Lebon Drive #202, San Diego, CA 92122 (US). DE LECEA, Luis [ES/US]; 13661 Nob Avenue, Del Mar, CA 92014 (US). BLOOM, Floyd, E. [US/US]; 628 Pacific View Drive, San Diego, CA 92109 (US). DANIELSON, Patria, E. [US/US]; 3436 Yonge Street, San Diego, CA 92106 (US). GAUTVIK, Vigdis, T. [NO/US]; 3365 Lebon Drive #202, San Diego, CA 92122 (US). KILDUFF, Thomas, S. [US/US]; 2660 Nansen Avenue, San Diego, CA 92122 (US). FOYE, Pamela, E.	[US/US]; 3946 Caminito del Mar Surf. San Diego, CA 92130 (US).  <b>(74) Agents:</b> OLSON, Amc, M.; Olson & Hierl, Ltd., 36th floor, 20 North Wacker Drive, Chicago, IL 60606 (US) et al.  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> HYPOTHALAMUS-SPECIFIC POLYPEPTIDES  <b>(57) Abstract</b>  <p>Directional tag PCR subtractive hybridization was used to construct a rat hypothalamic cDNA library from which cerebellar and hippocampal sequences had been depleted. Hypocretin, one of several novel hypothalamic-specific polypeptides identified, isolated and sequenced, is localized to regions of the hypothalamus involved in appetite and feeding behavior. Hypocretin polypeptides are biologically active, producing electrical changes in neurons, lowering body temperature and reducing food intake. The invention provides hypocretin polynucleotides and hypocretin polypeptides as well as antibodies, oligonucleotides, diagnostic kits and methods, and therapeutic compositions and methods.</p>		

## HYPOTHALAMUS-SPECIFIC POLYPEPTIDES

### Reference to Related Application

This application claims the benefit of U.S. Provisional Application S.N. 60/023,220, filed August 2, 1996, which is explicitly incorporated by  
5 reference, as are all references cited herein.

### Governmental Rights

This invention was made with governmental support from the United States Government, National Institutes of Health, Grants GM32355 and NS33396; the United States Government has certain rights in the invention.

### 10 Field of the Invention

This invention relates to the identification, isolation, sequencing, use, and expression of hypothalamus-specific proteins and fragments thereof.

### Background of the Invention

The hypothalamus, a phylogenetically ancient region of the  
15 mammalian brain, is responsible for the integration of the central nervous system and the endocrine system and is particularly related to the physiological response to stress. In contrast to laminar cortical structures such as the cerebellum and hippocampus whose final functions rely on innervation from the thalamus and brain stem, the hypothalamus is organized as a collection of distinct,  
20 autonomously active nuclei with discrete functions. Ablation and electrical stimulation studies and medical malfunctions have implicated several of these nuclei as central regulatory centers for major autonomic and endocrine homeostatic systems mediating processes such as reproduction, lactation, fluid balance, metabolism, and aspects of behaviors, such as circadian rhythmicity,  
25 basic emotions, feeding and drinking, mating activities, and responses to stress, as well as normal development of the immune system (Shepherd, G.M., *Neurobiology*, 3rd ed. Oxford University Press, New York, 1994). Distinct hormones and releasing factors have been associated with some of these nuclei but, at best, the organizations and molecular operations of these structures are  
30 only partially understood.

the polynucleotide of SEQ ID NO:4, a polynucleotide having at least about 95% of its nucleotide sequence identical to the polynucleotide of SEQ ID NO:3, and polynucleotides hybridizing to the polynucleotide of SEQ ID NO: 4.

Also provided are vectors for the expression of the novel  
5 polynucleotides operably linked to control sequences capable of directing the production of the novel polypeptides in suitable host cells.

In other aspects this invention provides pharmaceutical compositions of the polynucleotides, polypeptides and peptides, antibodies to the peptides and polypeptides as well as compositions thereof. This invention also  
10 provides assay methods and kits for practicing the methods, and methods for using the polynucleotides, peptides and polypeptides for diagnostic and therapeutic purposes.

#### Brief Description of the Drawings

In the Drawings,

15 Fig. 1 shows the results of subtractive screening, enriched for sequences selectively expressed in hypothalamus. Replicate dot blots on which the indicated masses of plasmid DNA for clones of neuron-specific enolase (NSE), cyclophilin, proopiomelanocortin (POMC), vasopressin, the vector pT7T3D, protein kinase C $\delta$  (PKC $\delta$ ) and growth hormone (GH) were manually  
20 spotted and hybridized with cDNA probes made from cRNA transcribed from the target or subtracted libraries, or an equal mixture of the cerebellum and hippocampus driver libraries. Comparison of the signal intensities for the vasopressin dilution series dots at several levels of autoradiographic exposure suggested a 20-to-30 fold increase in the specific activity of vasopressin cDNA.

25 Fig. 2. shows the results of cDNA library Southern blotting with clones representative of the four distribution classes. The electrophoretic lanes contain the cerebellum first driver library (D1), the hippocampus second driver library (D2), and the hypothalamus target library (T) cleaved with HaeIII and hybridized with the inserts from clone 35 (Panel A), clone 10 (Panel B), clone 86  
30 (Panel C) and clone 19 (Panel D).

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Fig. 7 is a graphical representation of the results of voltage clamp experiments on isolated in vitro rat hypothalamic cells, in which application of 1  $\mu$ g hcrt2 produced electrical responses in adult but not immature neurons.

#### Detailed Description of Preferred Embodiments

5           The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein. All patents and other publications mentioned in this specification are expressly incorporated by reference herein.

#### A. Definitions

10           Amino Acid Residue: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the  
15 polypeptide.  $\text{NH}_2$  refers to the free amino group present at the amino terminus of a polypeptide.  $\text{COOH}$  refers to the free carboxy group present at the carboxy terminus of a polypeptide. The standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 CFR §1.822(b)(2)) that provides one letter and three letter codes for amino acid  
20 residues is used.

It should be noted that all amino acid residue sequences represented herein by formulae have a left- to-right orientation in the conventional direction of amino terminus to carboxy terminus. In addition, the phrase "amino acid residue" is broadly defined to include modified and unusual amino acids,  
25 such as those listed in 37 CFR 1.822(b)(4), and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues or a covalent bond to an amino-terminal group such as  $\text{NH}_2$  or acetyl or to a carboxy-terminal group such as  $\text{COOH}$ .

30           Recombinant DNA molecule: a DNA molecule produced by

5' on the mRNA.

Downstream: Further along a DNA sequence in the direction of sequence transcription or read out, that is traveling in a 3'- to 5'-direction along the non-coding strand of the DNA or 5'- to 3'-direction along the RNA transcript.

Polypeptide: A linear series of amino acid residues connected to one another by peptide bonds between the alpha-amino group and carboxy group of contiguous amino acid residues.

Protein: A linear series of more than 50 amino acid residues connected one to the other as in a polypeptide.

Substantially Purified or Isolated: When used in the context of polypeptides or proteins, the terms describe those molecules that have been separated from components that naturally accompany them. Typically, a monomeric protein is substantially pure when at least about 60% to 75% of a sample exhibits a single polypeptide backbone. Minor variants or chemical modifications typically share the same polypeptide sequence. A substantially purified protein will typically comprise over about 85% to 90% of a protein sample, more usually about 95%, and preferably will be over about 99% pure. Protein or polypeptide purity or homogeneity may be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a sample, followed by visualization thereof by staining. For certain purposes, high resolution is needed and high performance liquid chromatography (HPLC) or a similar means for purification utilized.

Synthetic Peptide: A chemically produced chain of amino acid residues linked together by peptide bonds that is free of naturally occurring proteins and fragments thereof.

Nucleic acid or polynucleotide sequence: includes, but is not limited to, eucaryotic mRNA, cDNA, genomic DNA, and synthetic DNA and RNA sequences, comprising the natural nucleoside bases adenine, guanine, cytosine, thymidine, and uracil. The term also encompasses sequences having

control sequences so as to perform the desired function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. A coding sequence is operably linked to or under the control of transcriptional regulatory regions in a cell when DNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA that can be translated into the encoded protein. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

Heterologous and exogenous: as they relate to nucleic acid sequences such as coding sequences and control sequences, denote sequences that are not normally associated with a region of a recombinant construct, and are not normally associated with a particular cell. Thus, a "heterologous" region of a nucleic acid construct is an identifiable segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a construct could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Similarly, a host cell transformed with a construct which is not normally present in the host cell would be considered heterologous for purposes of this invention.

Expression system: polynucleotide sequences containing a desired coding sequence and control sequences in operable linkage, so that cells transformed with these sequences are capable of producing the encoded product. In order to effect transformation, the expression system may be included on a discrete vector; however, the relevant polynucleotide may also be integrated into the host chromosome.

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chloramphenicol acetyltransferase (CAT) and  $\beta$ -galactosidase ( $\beta$ -gal), may be incorporated into the primary nucleic acid cassette along with the gene expressing the desired therapeutic protein, or the selection markers may be contained on separate vectors and cotransfected.

- 5           The term "biochemically equivalent variations" means protein or nucleic acid sequences which differ in some respect from the specific sequences disclosed herein, but nonetheless exhibit the same or substantially the same functionality. In the case of cDNA, for example, this means that modified sequences which contain other nucleic acids than those specifically disclosed are
- 10 encompassed, provided that the alternate cDNA encodes mRNA which in turn encodes a protein of this invention. Such modifications may involve the substitution of only a few nucleic acids, or many. The modifications may involve substitution of degenerate coding sequences or replacement of one coding sequence with another; introduction of non-natural nucleic acids is included.
- 15 Preferably, the modified nucleic acid sequence hybridizes to and is at least 95% complementary to the sequence of interest.

- Similarly, in the case of the proteins and polypeptides of this invention, alterations in the amino acid sequence which do not affect functionality may be made. Such "biochemically equivalent muteins" may involve replacement
- 20 of one amino acid with another, use of side chain modified or non-natural amino acids, and truncation. The skilled artisan will recognize which sites are most amenable to alteration without affecting the basic function.

- The expression products described herein are proteins and polypeptides having a defined chemical sequence. However, the precise structure
- 25 depends on a number of factors, particularly chemical modifications common to proteins. For example, since all proteins contain ionizable amino and carboxyl groups, the protein may be obtained in acidic or basic salt form, or in neutral form. The primary amino acid sequence may be derivatized using sugar molecules (glycosylation) or by other chemical derivatizations involving covalent
- 30 or ionic attachment with, for example, lipids, phosphate, acetyl groups and the

Translation of this rat cDNA sequence produces a novel protein of 130 amino acid residues, referred to as rat preprohypocretin. The amino acid sequence of rat preprohypocretin is listed in SEQ ID NO: 1. The amino acid sequence of mouse preprohypocretin is listed in SEQ ID NO: 2.

5           A hypocretin protein of this invention can be in a variety of forms, depending upon the use therefor, as described herein. For example, a hypocretin can be isolated from a natural tissue.

          Alternatively, a hypocretin protein of this invention can be a recombinant protein, that is, produced by recombinant DNA methods as  
10       described herein. A recombinant hypocretin protein need not necessarily be substantially pure, or even isolated, to be useful in certain embodiments, although recombinant production methods are a preferred means to produce a source for further purification to yield an isolated or substantially pure receptor composition. A recombinant hypocretin protein can be present in or on a mammalian cell line  
15       or in crude extracts of a mammalian cell line.

          In one embodiment, a hypocretin protein is substantially free of other neuropeptides, so that the purity of a hypocretin reagent, and thus freedom from pharmacologically distinct proteins, facilitates use in the screening methods. The recombinant production methods are ideally suited to produce significantly  
20       improved purity in this regard, although biochemical purification methods from natural sources are also included. In this regard, a hypocretin protein is substantially free from other neuropeptides if there are insufficient other neuropeptides such that pharmacological cross-reactivity is not detected in conventional screening assays for ligand binding or biological activity.

25       Alternatively, recombinant hypocretin fusion proteins can be produced by joining nucleotides encoding additional amino acid residue sequence in proper reading frame at the 3' end of the hypocretin sequence. The fusion protein thus produced exhibits properties of the added amino acid sequence in addition to the properties of hypocretin. For example, the additional amino acid sequence may serve to  
30       help identify and purify the recombinantly produced hypocretin fusion protein.



(5)

invention includes a mammalian hypocretin protein, which can be derived, by recombinant DNA or biochemical purification from natural sources, from any of a variety of species including man, mouse, rabbit, rat, dog, cat, sheep, cow, and the like mammalian species, without limitation. Human and agriculturally  
5 relevant animal species are particularly preferred.

Exemplary hypocretin species identified herein are rat and mouse hypocretin.

The amino acid residue sequence of rat preprohypocretin is shown in SEQ ID NO 1, and corresponding nucleotide (cDNA) of rat preprohypocretin  
10 is shown in SEQ ID NO 3.

The amino acid residue sequence of mouse preprohypocretin is shown in SEQ ID NO 2, and corresponding nucleotide (cDNA) of mouse preprohypocretin is shown in SEQ ID NO 4.

A hypocretin protein of this invention can be prepared by a variety  
15 of means, although expression in a mammalian cell using a recombinant DNA expression vector is preferred. Exemplary production methods for a recombinant hypocretin are described in the Examples.

The invention also provides a method for the production of isolated hypocretin proteins, either as intact hypocretin protein, as fusion proteins or as  
20 smaller polypeptide fragments of hypocretin. The production method generally involves inducing cells to express a hypocretin protein of this invention, recovering the hypocretin from the resulting cells, and purifying the hypocretin so recovered by biochemical fractionation methods, using a specific antibody of this invention, or other chemical procedures.

25 The inducing step can comprise inserting a recombinant DNA vector encoding a hypocretin protein, or fragment thereof, of this invention, which recombinant DNA is capable of expressing a hypocretin, into a suitable host cell, and expressing the vector's hypocretin gene.

As used herein, the phrase "hypocretin polypeptide" refers to a  
30 polypeptide having an amino acid residue sequence that comprises an amino acid

preferred that a hypocretin polypeptide be no more than about 100 amino acid residues, still more preferably no more than about 50 residues, and optimally less than 40 amino acid residues in length when synthetic methods of production are used. Exemplary polypeptides are hcrt1 and hcrt2.

5           The present invention also includes a hypocretin polypeptide that has an amino acid residue sequence that corresponds to the sequence of the hypocretin protein shown in the sequence listings, and includes an amino acid residue sequence represented by a formula selected from the group consisting of the polypeptides shown in the sequence listings. In this embodiment, the  
10 polypeptide is further characterized as having the ability to mimic a hypocretin epitope and thereby inhibits hypocretin function in a classic hypocretin receptor activation assay, as described herein.

Due to the three dimensional structure of a native folded hypocretin molecule, the present invention includes that multiple regions of  
15 hypocretin are involved in hypocretin receptor function, which multiple and various regions are defined by the various hypocretin polypeptides described above. A preferred hypocretin receptor ligand is hcrt. The ability of the above-described polypeptides to inhibit receptor-ligand binding can readily be measured in a ligand binding assay as is shown in the Examples herein. Similarly, the  
20 ability of the above-described polypeptides to inhibit hypocretin receptor function can readily be measured in a receptor assay as is described herein.

In another embodiment, the invention includes hypocretin polypeptide compositions that comprise one or more of the different hypocretin polypeptides described above which inhibit hypocretin receptor function, admixed  
25 in combinations to provide simultaneous inhibition of multiple contact sites on the hypocretin receptor.

A subject polypeptide includes any analog, fragment or chemical derivative of a polypeptide whose amino acid residue sequence is shown herein so long as the polypeptide is capable of mimicking an epitope of hypocretin.  
30 Therefore, a present polypeptide can be subject to various changes, substitutions,

For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. D-amino acids may also be included in place of one or more L-amino acids. Polypeptides of the present invention also include any polypeptide having one or more additions and deletions or residues relative to the sequence of a polypeptide whose sequence is shown herein, so long as the requisite activity is maintained.

The term "fragment" refers to any subject polypeptide having an amino acid residue sequence shorter than that of a polypeptide whose amino acid residue sequence is shown herein.

When a polypeptide of the present invention has a sequence that is not identical to the sequence of a hypocretin polypeptide, it is typically because one or more conservative or non-conservative substitutions have been made, usually no more than about 30 number percent, more usually no more than 20 number percent, and preferably no more than 10 number percent of the amino acid residues are substituted. Additional residues may also be added at either terminus for the purpose of providing a "linker" by which the polypeptides of this invention can be conveniently affixed to a label or solid matrix, or carrier. Preferably the linker residues do not form a hypocretin epitope, i.e., are not similar in structure to a hypocretin protein.

Labels, solid matrices and carriers that can be used with the polypeptides of this invention are described hereinbelow.

Amino acid residue linkers are usually at least one residue and can be 40 or more residues, more often 1 to 10 residues, but do not form a hypocretin epitope. Typical amino acid residues used for linking are tyrosine, cysteine, lysine, glutamic and aspartic acid, or the like. In addition, a subject polypeptide can differ, unless otherwise specified, from the natural sequence of a hypocretin protein by the sequence being modified by terminal-NH<sub>2</sub> acylation, e.g., acetylation, or thioglycolic acid amidation, by terminal-carboxylamidation,

"Solid Phase Peptide Synthesis", W.H. Freeman Co., San Francisco, 1969; M. Bodansky, et al., "Peptide Synthesis", John Wiley & Sons, Second Edition, 1976 and J. Meienhofer, "Hormonal Proteins and Peptides", Vol. 2, p. 46, Academic Press (New York), 1983 for solid phase peptide synthesis, and E. Schroder and K. Kubke, "The Peptides", Vol. 1, Academic Press (New York), 1965 for classical solution synthesis, each of which is incorporated herein by reference. Additional peptide synthesis methods are described by Sutcliffe in U.S. Patent No. 4,900,811 and 5,242,798, which are hereby incorporated by reference. Appropriate protective groups usable in such synthesis are described in the above texts and in J.F.W. McOmie, "Protective Groups in Organic Chemistry", Plenum Press, New York, 1973, which is incorporated herein by reference.

In general, the solid-phase synthesis methods comprise the sequential addition of one or more amino acid residues or suitably protected amino acid residues to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid residue is protected by a suitable, selectively removable protecting group. A different, selectively removable protecting group is utilized for amino acids containing a reactive side group such as lysine.

Using a solid phase synthesis as exemplary, the protected or derivatized amino acid is attached to an inert solid support through its unprotected carboxyl or amino group. The protecting group of the amino or carboxyl group is then selectively removed and the next amino acid in the sequence having the complimentary (amino or carboxyl) group suitably protected is admixed and reacted under conditions suitable for forming the amide linkage with the residue already attached to the solid support. The protecting group of the amino or carboxyl group is then removed from this newly added amino acid residue, and the next amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining terminal and side group protecting groups (and solid support) are removed sequentially or concurrently, to afford the final polypeptide.

amino acid residue. Therefore, a number of different nucleotide sequences may code for a particular amino acid residue sequence. Such nucleotide sequences are considered functionally equivalent since they can result in the production of the same amino acid residue sequence in all organisms. Occasionally, a methylated variant of a purine or pyrimidine may be incorporated into a given nucleotide sequence. However, such methylations do not affect the coding relationship in any way.

A nucleic acid is any polynucleotide or nucleic acid fragment, whether it be a polyribonucleotide or polydeoxyribonucleotide, i.e., RNA or DNA, or analogs thereof. In preferred embodiments, a nucleic acid molecule is in the form of a segment of duplex DNA, i.e., a DNA segment, although for certain molecular biological methodologies, single-stranded DNA or RNA is preferred.

DNA segments (i.e., synthetic oligonucleotides) that encode portions of hypocretin proteins can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, et al., (J. Am. Chem. Soc., 103:3185-3191, 1981) or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define the DNA segment, followed by hybridization and ligation of oligonucleotides to build the complete segment.

Of course, by chemically synthesizing the coding sequence, any desired modifications can be made simply by substituting the appropriate bases for those encoding the native amino acid residue sequence.

Furthermore, DNA segments consisting essentially of structural genes encoding a hypocretin protein can be obtained from recombinant DNA molecules containing a gene that defines a hypocretin protein of this invention, and can be subsequently modified, as by site directed mutagenesis, to introduce any desired substitutions.

#### 1. Cloning Hypocretin Genes

Hypocretin genes of this invention can be cloned by a variety of

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repeats are present in either normal, underexpansion or overexpansion form can provide a genetic basis for diagnosis for some diseases. The same may be true for hypocretin in that expansion of the region may contribute to the basis for a neuronal disorder or disease of the brain or other tissue.

5                   2.   Oligonucleotides

The invention also includes oligonucleotides useful for methods to detect the presence of a hypocretin gene or gene transcript (mRNA) in a tissue by diagnostic detection methods based on the specificity of nucleic acid hybridization or primer extension reactions. One embodiment includes any polynucleotide  
10 probe having a sequence of a portion of a hypocretin gene of this invention, or a related and specific sequence. Hybridization probes can be of a variety of lengths from about 10 to 5000 nucleotides long, although they will typically be about 20 to 500 nucleotides in length. Hybridization methods are extremely well known in the art and will not be described further here.

15                   In a related embodiment, detection of hypocretin genes can be conducted by primer extension reactions such as the polymerase chain reaction (PCR). To that end, PCR primers are utilized in pairs, as is well known, based on the nucleotide sequence of the gene to be detected. Particularly preferred PCR primers can be derived from any portion of a hypocretin DNA sequence,  
20 but are preferentially from regions which are not conserved in other cellular proteins.

A preferred PCR primer pair useful for detecting hypocretin genes and hypocretin gene expression are described in the Examples. Nucleotide primers from the corresponding region of hypocretin described herein are readily  
25 prepared and used as PCR primers for detection of the presence or expression of the corresponding gene in any of a variety of tissues.

3.   Expression Vectors

In addition, the invention includes a recombinant DNA molecule (recombinant DNA) containing a DNA segment of this invention encoding a  
30 hypocretin protein as described herein. A recombinant DNA can be produced by

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those compatible with vertebrate cells, can also be used to form the recombinant DNA molecules of the present invention. Eucaryotic cell expression vectors are well known in the art and are available from several commercial sources.

Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), pRc/CMV (Invitrogen, Inc.); the vector pCMV4 described herein, and the like eucaryotic expression vectors.

In preferred embodiments, the eucaryotic cell expression vectors used to construct the recombinant DNA molecules of the present invention include a selection marker that is effective in an eucaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, i.e., the neomycin phosphotransferase (neo) gene. Southern et al., *J. Mol. Appl. Genet.*, 1:327-341 (1982). Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by co-transfection of the host cell, and selected by culturing in the appropriate drug for the selectable marker.

#### 4. Inhibitory Nucleic Acids

In accordance with one embodiment of the invention, nucleic acid molecules can be used in methodologies for the inhibition of hypocretin gene expression, thereby inhibiting the function of the hypocretin: hypocretin receptor binding interaction by blocking hypocretin expression.

To that end, the invention includes isolated nucleic acid molecules, preferably single-stranded nucleic acid molecules (oligonucleotides), having a sequence complementary to a portion of a structural gene encoding a hypocretin protein of this invention. Nucleic acid-based inhibition is well known and generally referred to as "anti-sense" technology by virtue of the use of nucleotide sequences having complementarity which can hybridize to the "sense" strand or mRNA, and thereby perturb gene expression. Typical oligonucleotides for this purpose are about 10 to 5,000, preferably about 20-1000, nucleotides in

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nucleotide sequence can be substituted with a structure which functions to enhance the ability of the compositions to penetrate into the region of cells where the hypocretin structural gene to be inhibited is located. It is preferred that such linkages be sulfur containing as discussed above, such as phosphorothioate bonds.

- 5 Other substitutions can include alkyl phosphothioate bonds, N-alkyl phosphoramidates, phosphorodithioates, alkyl phosphonates, and short chain alkyl or cycloalkyl structures. In accordance with other preferred embodiments, the phosphodiester bonds are substituted with structures which are, at once, substantially non-ionic and non-chiral.

10 D. Anti-Hypocretin Antibodies

An antibody of the present invention, i.e., an anti-hypocretin antibody, in one embodiment is characterized as comprising antibody molecules that immunoreact with a hypocretin protein of this invention. Preferably, an antibody further immunoreacts with a hypocretin protein in situ, i.e., in a tissue  
15 section.

The invention describes an anti-hypocretin antibody that immunoreacts with any of the hypocretin polypeptides of this invention, preferably also immunoreacts with the corresponding recombinant hypocretin protein, and more preferably also reacts with a native protein in situ in a tissue  
20 section. Preferably, the antibody is substantially free from immunoreaction with other proteins or neuropeptides other than hypocretin. Assays for immunoreaction useful for assessing immunoreactivity are described herein.

In one embodiment, antibody molecules are described that immunoreact with a hypocretin receptor polypeptide of the present invention and  
25 that have the capacity to immunoreact with an exposed site on hypocretin that is required for hypocretin receptor binding. Thus, preferred antibody molecules in this embodiment also inhibit hypocretin receptor function, and are therefore useful therapeutically to block the receptor's function.

Exemplary hypocretin inhibitory antibodies immunoreact with a  
30 hypocretin polypeptide described herein that defines an exposed region of a



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The word "inoculum" in its various grammatical forms is used herein to describe a composition containing a hypocretin polypeptide of this invention as an active ingredient used for the preparation of antibodies against a hypocretin polypeptide. When a polypeptide is used in an inoculum to induce antibodies it is to be understood that the polypeptide can be used in various embodiments, e.g., alone or linked to a carrier as a conjugate, or as a polypeptide polymer. However, for ease of expression and in context of a polypeptide inoculum, the various embodiments of the polypeptides of this invention are collectively referred to herein by the term "polypeptide" and its various grammatical forms.

For a polypeptide that contains fewer than about 35 amino acid residues, it is preferable to use the peptide bound to a carrier for the purpose of inducing the production of antibodies.

One or more additional amino acid residues can be added to the amino- or carboxy-termini of the polypeptide to assist in binding the polypeptide to a carrier. Cysteine residues added at the amino- or carboxy-termini of the polypeptide have been found to be particularly useful for forming conjugates via disulfide bonds. However, other methods well known in the art for preparing conjugates can also be used.

The techniques of polypeptide conjugation or coupling through activated functional groups presently known in the art are particularly applicable. See, for example, Aurameas, et al., Scand. J. Immunol., Vol. 8, Suppl. 7:7-23 (1978) and U.S. Patent No. 4,493,795, No. 3,791,932 and No. 3,839,153. In addition, a site-directed coupling reaction can be carried out so that any loss of activity due to polypeptide orientation after coupling can be minimized. See, for example, Rodwell et al., Biotech., 3:889-894 (1985), and U.S. Patent No. 4,671,958.

Exemplary additional linking procedures include the use of Michael addition reaction products, di-aldehydes such as glutaraldehyde, Klipstein, et al., J. Infect. Dis., 147:318-326 (1983) and the like, or the use of carbodiimide

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achieved, and (b) the limitations inherent in the art of compounding such active material for immunologic use in animals, as disclosed in detail herein, these being features of the present invention.

5 Inocula are typically prepared from the dried solid polypeptide-conjugate by dispersing the polypeptide-conjugate in a physiologically tolerable (acceptable) diluent such as water, saline or phosphate-buffered saline to form an aqueous composition.

Inocula can also include an adjuvant as part of the diluent. Adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's  
10 adjuvant (IFA) and alum are materials well known in the art, and are available commercially from several sources.

The antibody so produced can be used, inter alia, in the diagnostic methods and systems of the present invention to detect hypocretin present in a sample such as a tissue section or body fluid sample. Anti-hypocretin antibodies  
15 that inhibit hypocretin function can also be used in vivo in therapeutic methods as described herein.

A preferred anti-hypocretin antibody is a monoclonal antibody. A preferred monoclonal antibody of this invention comprises antibody molecules that immunoreact with a hypocretin polypeptide of the present invention as  
20 described for the anti-hypocretin antibodies of this invention. More preferably, the monoclonal antibody also immunoreacts with recombinantly produced whole hypocretin protein.

A monoclonal antibody is typically composed of antibodies produced by clones of a single cell called a hybridoma that secretes (produces)  
25 only one kind of antibody molecule. The hybridoma cell is formed by fusing an antibody-producing cell and a myeloma or other self-perpetuating cell line. The preparation of such antibodies was first described by Kohler and Milstein, Nature, 256:495-497 (1975), the description of which is incorporated by reference. The hybridoma supernates so prepared can be screened for the  
30 presence of antibody molecules that immunoreact with a hypocretin polypeptide,

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media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's Minimal Essential Medium (DMEM; Dulbecco et al., Virology 8:396 (1959)) supplemented with 4.5 gm/l glucose, 20 mM glutamine, and 20% fetal calf serum. An exemplary inbred mouse strain is the Balb/c.

5 Other methods of producing a monoclonal antibody, a hybridoma cell, or a hybridoma cell culture are also well known. See, for example, the method of isolating monoclonal antibodies from an immunological repertoire as described by Sastry, et al., Proc. Natl. Acad. Sci. USA, 86:5728-5732 (1989); and Huse et al., Science, 246:1275-1281 (1989).

10 The monoclonal antibodies of this invention can be used in the same manner as disclosed herein for antibodies of the present invention.

For example, the monoclonal antibody can be used in the therapeutic, diagnostic or in vitro methods disclosed herein where immunoreaction with hypocretin is desired.

15 Also included in this invention is the hybridoma cell, and cultures containing a hybridoma cell that produce a monoclonal antibody of this invention.

#### E. Diagnostic Methods

The present invention includes various assay methods for determining the presence, and preferably amount, of hypocretin in a body sample  
20 such as a tissue sample, including tissue mass or tissue section, or in a biological fluid sample using a polypeptide, polyclonal antibody or monoclonal antibody of this invention as an immunochemical reagent to form an immunoreaction product whose amount relates, either directly or indirectly, to the amount of hypocretin in the sample.

25 Those skilled in the art will understand that there are numerous well known clinical diagnostic chemistry procedures in which an immunochemical reagent of this invention can be used to form an immunoreaction product whose amount relates to the amount of hypocretin in a body sample. Thus, while exemplary assay methods are described herein, the invention is not so limited.

30 For example, in view of the demonstrated property that hypocretin

described for well known Western blot procedures.

- (b) The immunoreaction admixture is maintained under biological assay conditions for a predetermined time period such as about 10 minutes to about 16-20 hours at a temperature of about 4 degree Celsius to about 45 degree Celsius that, such time being sufficient for the hypocretin present in the sample to immunoreact with (immunologically bind) the antibody and form a hypocretin-containing immunoreaction product (immunocomplex).

Biological assay conditions are those that maintain the biological activity of the immunochemical reagents of this invention and the hypocretin sought to be assayed. Those conditions include a temperature range of about 4 degree Celsius to about 45 degree Celsius, a pH value range of about 5 to about 9 and an ionic strength varying from that of distilled water to that of about one molar sodium chloride. Methods for optimizing such conditions are well known in the art.

- (c) The presence, and preferably amount, of hypocretin-containing immunoreaction product that formed in step (b) is determined (detected), thereby determining the amount of hypocretin present in the sample.

Determining the presence or amount of the immunoreaction product, either directly or indirectly, can be accomplished by assay techniques well known in the art, and typically depend on the type of indicating means used.

Preferably, the determining of step (c) comprises the steps of:

- (i) admixing the hypocretin-containing immunoreaction product with a second antibody to form a second (detecting) immunoreaction admixture, said second antibody molecule having the capacity to immunoreact with the first antibody (primary) in the immunoreaction product.

Antibodies useful as the second antibody include polyclonal or monoclonal antibody preparations raised against the primary antibody.

- (ii) maintaining said second immunoreaction admixture for a time period sufficient for said second antibody to complex with the immunoreaction product and form a second immunoreaction product, and

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(a) Forming a competition immunoreaction admixture by admixing (contacting) a fluid sample with:

5 (1) an anti-hypocretin antibody according to this invention containing antibody molecules that immunoreact with a hypocretin protein of this invention, said antibody being operatively linked to a solid matrix such that the competition immunoreaction admixture has both a liquid phase and a solid phase, and

10 (2) a polypeptide or recombinant hypocretin protein of the present invention that is immunoreactive with the added antibody. The admixed polypeptide/protein in the liquid phase (labeled competing antigen) is operatively linked to an indicating means as described herein.

15 (b) The competition immunoreaction admixture is then maintained for a time period sufficient for the competing antigen and the body sample antigen present in the liquid phase to compete for immunoreaction with the solid phase antibody. Such immunoreaction conditions are previously described, and result in the formation of an indicating means-containing immunoreaction product comprising the labeled competing antigen in the solid phase.

20 (c) The amount of indicating means present in the product formed in step (b) is then determined, thereby determining the presence, and preferably amount, of sample antigen present in the fluid sample.

Determining the indicating means in the solid phase is then conducted by the standard methods described herein.

A reverse version of this embodiment comprises the steps of:

25 (a) Forming a competition immunoreaction admixture by admixing a fluid sample with:

(1) an anti-hypocretin antibody according to the present invention; and

30 (2) a hypocretin polypeptide or recombinant hypocretin protein of the present invention (capture antigen) that is immunoreactive with the

hypocretin while present in the hypocretin-antibody immunoreaction complex. This embodiment can be practiced in two formats with the immobilized capture antibody being either of the two above-identified antibodies, and the indicator antibody being the other of the two antibodies.

5               Where an antibody is in the solid phase as a capture reagent, a preferred means for determining the amount of solid phase reaction product is by the use of a labeled hypocretin polypeptide, followed by the detection means described herein for other labeled products in the solid phase.

10               Also included are immunological assays capable of detecting the presence of immunoreaction product formation without the use of a label. Such methods employ a "detection means", which means are themselves well-known in clinical diagnostic chemistry and constitute a part of this invention only insofar as they are utilized with otherwise novel polypeptides, methods and systems. Exemplary detection means include methods known as biosensors and include  
15               biosensing methods based on detecting changes in the reflectivity of a surface, changes in the absorption of an evanescent wave by optical fibers or changes in the propagation of surface acoustical waves.

                 Alternative methods of expression, amplification, and purification will be apparent to the skilled artisan. Representative methods are disclosed in  
20               Sambrook, Fritsch, and Maniatis, eds. *Molecular Cloning, a Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory (1989) and in Ausabel et al., eds., *Current Protocols in Molecular Biology*, Wiley & Sons, Inc., New York (1989).

D.   Specific Methods

                 Directional tag PCR subtractive hybridization was used to enrich a  
25               cDNA library for clones of mRNA species selectively expressed in the hypothalamus. Candidate clones identified by their hybridization to a subtracted hypothalamus probe were validated in three stages. First, a high throughput cDNA library Southern blot was used to demonstrate that the candidate corresponded to a species enriched in the subtracted library. Second, candidate  
30               clones positive in the first assay were used as probes for Northern blots with

digested with HaeIII, separated by electrophoresis, transferred to nylon membranes, and hybridized to individual clones, as described in Usui et al, *supra*.

To recognize mRNAs that are selectively expressed in the hypothalamus, poly(A)-enriched cytoplasmic RNA from carefully dissected rat and mouse hypothalami were prepared. Target cDNA libraries in vector pT7T3D (Pharmacia Biotech, Piscataway, NJ) and driver libraries in pGEM11Zf(-) (Promega, Madison, WI) from analogously prepared cerebellar and hippocampal RNA samples were constructed. The directional tag PCR subtractive hybridization method of Usui and colleagues in Usui et al., *supra* was applied to produce tagged hypothalamic cDNAs from which cerebellar and hippocampal sequences were depleted in two consecutive steps, removing more than 97% of the input target cDNA. The tag sequences were used as PCR primer-binding sites to amplify the remaining material. An aliquot of the amplified product was cloned into pBCSK<sup>+</sup> (Stratagene, La Jolla, CA) to generate a subtracted hypothalamus library with  $5 \times 10^5$  members, with inserts ranging from 400 to 1200 (average 700) nucleotide pairs, as judged by agarose gel electrophoresis of the released inserts.

To validate the efficiency of the subtraction, the degree of depletion in the subtracted library of sequences known to be expressed panneurally and the enrichment of sequences known to be expressed specifically in the hypothalamus was determined. Dot blots were prepared with dilutions of cDNA clones of the mRNAs encoding the following proteins: panneural neuron-specific enolase, ubiquitously expressed cyclophilin, hypothalamus-specific vasopressin, hypothalamus-enriched proopiomelanocortin (POMC), thalamus-specific protein kinase C $\delta$ , and pituitary-specific growth hormone, as well as the target vector itself. The blots were probed with cDNA inserts amplified by PCR from the unsubtracted target library, the subtracted target library or a pool of the driver libraries (Fig. 1). The driver and unsubtracted-library probes gave strong signals for cyclophilin and neuron-specific enolase, and a weaker signal for

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using a sequencing primer that annealed to the vector region adjacent to the 3' ends of the inserts. The remaining 6 clones were not pursued further because clear sequences were not obtained. More than 90% of the 3' sequences appeared to be derived from bona fide 3' ends of mRNAs as they contained recognizable poly(A)-addition consensus hexads (Birnstiel, M.L., Busslinger, M., & Strub, K. *Cell* 41:349-359, 1985) 12-22 nucleotides upstream from the poly(A) tracts used in their directional cloning. The sequences were searched by BLAST analysis (Altschul, S.F., Gish, W., Miller, W., Myers, E.W., & Lipman, D., *J. Molec. Biol.* 215:403, 1990) against the GenBank database. For those that appeared to be novel, the sequence at the 5' end of the insert was also determined and compared with the database.

A compilation of those data is presented in Table 1 and database accession numbers are given for those prototypes for which a match was found. The 94 clones from the subtracted library for which data were obtained corresponded to 43 distinct mRNA species. Twenty-nine of these were encountered only once in the set of 94 clones, while 14 species were seen between 2 to 13 times. Among the 43 distinct species were 21 that were unambiguously matched to known mRNA species and 22 that were novel species. Amongst the novel species were 6 that appear to correspond (greater than 80% nucleotide sequence identity across an extensive span) to rat homologues of so-called "expressed sequence tags" (ESTs), mRNAs of as yet unknown function compiled in the databases. Two species exhibited similarities in both their partial nucleotide sequences and putative encoded amino acid sequences that suggest them to represent members of protein families: a protein related to the VAT-1 secretory vesicle protein (clone 6), and a new calmodulin-dependent protein kinase (clone 29, SEQ ID NO:5).

The cDNA insert from at least one representative of each of the 43 mRNA species was used as a probe in a Southern blot with lanes corresponding to the hypothalamus, hippocampus and cerebellum target and driver cDNA libraries, each cleaved with the restriction endonuclease HaeIII. Assuming that



blot study, each detected a band that was strong in the hypothalamus lanes, but only very faint or undetectable in the other lanes. The faint signals were possibly due to low expression in those tissues or to contamination during tissue dissection. Clones 6 (VAT1-like), 10 (novel) and 12 (novel), which had given B patterns, each detected bands that were considerably more intense in the hypothalamus than hippocampus or cerebellum lanes, although each was detected in the pituitary lane (6 strongly) and in the samples from some other structures. Clones 3 (novel), 15 (novel) and 29 (novel calmodulin-dependent protein kinase), although classified originally as B patterns, are more properly considered as C patterns, as their expression profiles in this assay are not enriched in hypothalamus per se, but rather are low in the cerebellum.

The clones encountered only once behaved, as a group, less well. Clones 21 (novel), 37 (novel), 98 (novel) and 99 (kinesin) failed to show substantial enrichment in hypothalamus over hippocampus or cerebellum (although 98 was thalamus enriched). However, clone 33 (novel) detected an RNA species more prevalent in hypothalamus and thalamus than cortex, pons or olfactory bulb and was undetectable in hippocampus, cerebellum or peripheral tissues; thus, technically speaking, clone 33 maintained its A pattern classification. Clone 20 (novel) detected an RNA species with ubiquitous expression but enrichment in hypothalamus and thalamus, thus it is more properly classified as B pattern. Clone 67 (novel) detected a species enriched in hypothalamus and olfactory bulb that was detectable in other brain regions and pituitary but was not detectable in cerebellum.

In situ hybridization on coronal sections of brain from adult male rats was performed using the inserts from clones representing all four classes (A-D): 6, 10, 20, 21, 29 and 35. For all clones, the hybridization pattern was consistent with the Northern blot data. In the A class, the clone 35 mRNA displayed a striking pattern of bilaterally symmetric expression restricted to a few cells in the paraventricular hypothalamic area and ependymal cells surrounding the brain ventricles. No clone 35 signals were detected outside the hypothalamus.

higher in the hybridization reaction, thus a greater portion of the common species were driven into hybrids. Cumulatively, 85 of the 94 candidates were found to be enriched in the target hypothalamus compared to the cerebellum, a quite acceptable success rate. It is noteworthy that in 8 cases, the cDNA library Southern blot assay suggested a higher degree of hypothalamus enrichment than was later observed by Northern blotting, presumably due to artifactual enrichment in the target libraries compared to the driver libraries. In a few cases this can be explained by artifactual cloning of an internal or intronic cDNA fragment. Other cases may be explained by difficulties in achieving proportional representation of low prevalence mRNAs in cDNA libraries.

The subtraction steps provided an approximately 30-fold enrichment. In the secondary screen, approximately 60% of the clones were positive with the subtracted probe but not the target probe. Of the 94 clones selected from this screen, 53 were clones of mRNAs selectively expressed in hypothalamus. These 53 clones correspond to approximately 1% of the clones examined in this pilot study, and represented 16 distinct mRNA species, suggesting that a complete characterization of hypothalamus mRNAs might reveal 100-200 species that were specific to or highly enriched in the hypothalamus. Of the 16 mRNA species detected here, 9 corresponded to already known proteins, among them oxytocin, vasopressin and POMC, three neuropeptides known to be highly enriched in the hypothalamus. However, 7 mRNA species were novel. Among mRNA species not detected in the 94-clone sample were those encoding the releasing factors, which are less abundant than most of the species detected here.

Oxytocin and vasopressin mRNAs are predominantly associated with discrete hypothalamic nuclei, as was previously known. The in situ hybridization images indicate that several additional mRNAs, including several novel species, are enriched in the hypothalamus. Among the novel species, only clone 35 meets the hypothesis in its strictest sense: the mRNA appears to be restricted to nuclei in the paraventricular area of the hypothalamus.

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differences, 19 nucleotides differ within the protein coding region. Only 7 of these affect the encoded protein sequence. One amino acid difference is a neutral substitution in the secretion signal sequence (residue 3). The remaining 6 differences are in the C-terminal region. One of these obliterates a potential proteolytic cleavage site. This observation and the nature of the other differences make it unlikely that 2 of the possible maturation products of the rat preproprotein are functional. However, the 2 peptides that are related both to each other and to secretin are absolutely preserved between species, providing strong support for the notion that these peptides have a function conserved during evolution.

The cells that express this mRNA are distributed in a bilaterally symmetrical pattern in a previously uncharted nucleus of the rat dorsal-lateral hypothalamus and sparse ependymal cells that line the ventricles suggesting that the peptides function as intercellular messengers within the CNS. Colocalization studies suggest a partial overlap with cells positive for galanin, bradykinin and dynorphin. The rat H35 mRNA is restricted to the CNS in the studies performed to date. It is not expressed at high concentrations in immature animals.

These observations, along with the sequence data discussed above, suggest that the H35 peptides are secreted into the CSF and locally within the hypothalamus; that their functions are only manifested in mature animals; and that their expression is coupled to the general homeostatic status of the animal, although not regulated in an all-or-none fashion by homeostasis. In other words, these are new hormones that act within the central nervous system.

The polypeptides may be expressed by transformation of a suitable host cell with a cDNA in a suitable expression vector. The choice of host cell is not critical. The polypeptide may be produced from a procaryotic (e.g. *E. coli*) or eucaryotic (mammalian, e.g. COS-7, CHO, NIH 3T3) host cell, as desired.

The hypocretin polypeptides, and fragments thereof, of this invention are useful in diagnosis and therapy. Recombinant or natural polypeptides may be used in Western blot, ELISA, RIA, and the like, and in

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and the physical condition and mental acuity of the intended recipient. Such considerations are within the purview of the skilled artisan.

Representative delivery regimens include oral, parenteral (subcutaneous, intramuscular, and intravenous), rectal, buccal, pulmonary, transdermal, and intranasal, preferably intravenous. The composition may be in solid, liquid, gel, or aerosol form. Generally, the compound will be present in an amount from about 1  $\mu\text{g}$  to about 100  $\mu\text{g}$ , in a sterile aqueous solution, optionally including stabilizers and the like.

The present invention also describes a diagnostic system, preferably in kit form, for assaying for the presence of a hypocretin of this invention in a body sample, such brain tissue, cell suspensions or tissue sections, or body fluid samples such as CSF, blood, plasma or serum, where it is desirable to detect the presence, and preferably the amount, of a hypocretin protein in the sample according to the diagnostic methods described herein.

In a related embodiment, a nucleic acid molecule can be used as a probe (an oligonucleotide) to detect the presence of a gene or mRNA in a cell that is diagnostic for the presence or expression of a hypocretin in the cell. The nucleic acid molecule probes were described in detail earlier.

The diagnostic system includes, in an amount sufficient to perform at least one assay, a subject hypocretin polypeptide, a subject antibody or monoclonal antibody, and a subject nucleic acid molecule probe of the present invention, as a separately packaged reagent.

Another embodiment is a diagnostic system, preferably in kit form, for assaying for the presence of a hypocretin polypeptide or anti-hypocretin antibody in a body fluid sample such as for monitoring the fate of therapeutically administered hypocretin polypeptide or anti-hypocretin antibody. The system includes, in an amount sufficient for at least one assay, a subject hypocretin polypeptide and a subject antibody as a separately packaged immunochemical reagent.

Instructions for use of the packaged reagent(s) are also typically

clinical diagnostic chemistry and constitute a part of this invention only insofar as they are utilized with otherwise novel proteins methods and systems.

The labeling means can be a fluorescent labeling agent that chemically binds to antibodies or antigens without denaturing them to form a fluorochrome (dye) that is a useful immunofluorescent tracer. Suitable fluorescent labeling agents are fluorochromes such as fluorescein isocyanate (FIC), fluorescein isothiocyanate (FITC), 5-diethylamine-1-naphthalenesulfonyl chloride (DANSC), tetramethylrhodamine isothiocyanate (TRITC), lissamine, rhodamine 8200 sulphonyl chloride (RB 200 SC) and the like. A description of immunofluorescence analysis techniques is found in DeLuca, "Immunofluorescence Analysis", in Antibody As a Tool, Marchalonis, et al., eds., John Wiley & Sons, Ltd., pp. 189-231 (1982), which is incorporated herein by reference.

In preferred embodiments, the indicating group is an enzyme, such as horseradish peroxidase (HRP), glucose oxidase, or the like. In such cases where the principal indicating group is an enzyme such as HRP or glucose oxidase, additional reagents are required to visualize the fact that a receptor-ligand complex (immunoreactant) has formed. Such additional reagents for HRP include hydrogen peroxide and an oxidation dye precursor such as diaminobenzidine. An additional reagent useful with glucose oxidase is 2,2'-amino-di-(3-ethyl-benzthiazoline-G-sulfonic acid) (ABTS).

Radioactive elements are also useful labeling agents and are used illustratively herein. An exemplary radiolabeling agent is a radioactive element that produces gamma ray emissions. Elements which themselves emit gamma rays, such as  $^{124}\text{I}$ ,  $^{125}\text{I}$ ,  $^{128}\text{I}$ ,  $^{132}\text{I}$  and  $^{51}\text{Cr}$  represent one class of gamma ray emission-producing radioactive element indicating groups. Particularly preferred is  $^{125}\text{I}$ . Another group of useful labeling means are those elements such as  $^{11}\text{C}$ ,  $^{18}\text{F}$ ,  $^{15}\text{O}$  and  $^{13}\text{N}$  which themselves emit positrons. The positrons so emitted produce gamma rays upon encounters with electrons present in the animal's body. Also useful is a beta emitter, such as  $^{111}\text{In}$  or  $^3\text{H}$ .

Publications of Los Altos, CA in 1982 and in U.S. Patents No. 3,654,090; No. 3,850,752; and No. 4,016,043, which are all incorporated herein by reference.

In some embodiments, a hypocretin polypeptide, an antibody or a monoclonal antibody of the present invention can be affixed to a solid matrix to form a solid support that comprises a package in the subject diagnostic systems.

A reagent is typically affixed to a solid matrix by adsorption from an aqueous medium although other modes of affixation applicable to proteins and polypeptides can be used that are well known to those skilled in the art.

Exemplary adsorption methods are described herein.

Useful solid matrices are also well known in the art. Such materials are water insoluble and include the cross-linked dextran available under the trademark SEPHADEX from Pharmacia Fine Chemicals (Piscataway, NJ); agarose; beads of polystyrene beads about 1 micron ( $\mu$ ) to about 5 millimeters (mm) in diameter available from Abbott Laboratories of North Chicago, IL; polyvinyl chloride, polystyrene, cross-linked polyacrylamide, nitrocellulose- or nylon-based webs such as sheets, strips or paddles; or tubes, plates or the wells of a microliter plate such as those made from polystyrene or polyvinylchloride.

The reagent species, labeled specific binding agent or amplifying reagent of any diagnostic system described herein can be provided in solution, as a liquid dispersion or as a substantially dry power, e.g., in lyophilized form. Where the indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package of a system. A solid support such as the before-described microliter plate and one or more buffers can also be included as separately packaged elements in this diagnostic assay system.

The packaging materials discussed herein in relation to diagnostic systems are those customarily utilized in diagnostic systems.

#### G. Cell Lines Expressing Hypocretin

The invention also includes a host cell transformed with a recombinant DNA (recombinant DNA) molecule of the present invention. The host cell can be either procaryotic or eucaryotic, although eucaryotic cells are

CRL 1573), Ltk-1, AV-12 (ATCC CRL 9595), and the like eucaryotic tissue culture cell lines.

Transformation of appropriate cell hosts with a recombinant DNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of procaryotic host cells, see, for example, Cohen et al., Proc. Natl. Acad. Sci. USA, 69:2110 (1972); and Maniatis et al., Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

With regard to transformation of vertebrate cells with vectors containing recombinant DNAs, see, for example, Graham et al., Virology, 52:456 (1973); Wigler et al., Proc. Natl. Acad. Sci. USA, 76:1373-76 (1979), and the teachings herein.

Successfully transformed cells, i.e., cells that contain a recombinant DNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an recombinant DNA of the present invention can be cloned to clonally homogeneous cell populations that contain the recombinant DNA. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the recombinant DNA using a method such as that described by Southern, J. Mol. Biol., 98:503 (1975) or Berent et al., Biotech., 3:208 (1985).

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of hypocretin or by the detection of hypocretin binding activity.

For example, cells successfully transformed with an expression vector produce proteins displaying hypocretin antigenicity or biological activity. Samples of cells suspected of being transformed are harvested and assayed for either hypocretin biological activity or antigenicity.

In addition to the transformed host cells themselves, the present invention also includes a culture of those cells, preferably a monoclonal (clonally

(c)

label in the presence of a candidate ligand, indicating whether the candidate competes with labeled hypocretin for binding to the receptor. An exemplary competition assay is described herein.

5 It is also possible to use the above method to determine whether the molecule which binds to the hypocretin receptor also activates or motivates the receptor's function, i.e., acts as an agonist, or determine whether the molecule inhibits the receptor's function, i.e., acts as an antagonist, or acts as and inverse agonist. Thus, by evaluating in the detecting step whether the hypocretin receptor is activated, one determines whether the candidate molecule is  
10 bioactive.

Methods for detecting bioactivity of the candidate molecule can vary, but typically involve measuring changes in intracellular levels of a secondary messenger effected as a result of binding, detecting changes in electrical potential, observing physiological or behavioral effects related to  
15 hypocretin function, and the like methods. Exemplary assays for binding or for hypocretin-specific bioactivity are described in the Examples and include measurement of electrical changes of hypothalamic neurons, measurement of food intake or body temperature, or direct binding to a cell having a hypocretin receptor.

20 It is noted that the hypocretin receptor has not been characterized in extensive detail. Thus, any receptor that binds hypocretin can be referred to as a hypocretin receptor for the purposes of a screening assay, although receptors with the highest affinity and specificity for hypocretin are preferred. In practicing the present screening methods, one can use any of a variety of cells  
25 lines or tissues that possess a hypocretin receptor, including the exemplary cell lines and tissues described herein. The invention should not be construed as limiting so long as the binding or bioactivity assay involves the use of a hypocretin receptor. In preferred embodiments, a receptor that is specific for hypocretin should be used. Specificity can be demonstrated by well known  
30 methods of ligand binding and ligand-mediated activation.



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tolerable composition containing a hypocretin polypeptide, analog or peptidomimetic, anti-hypocretin antibody or monoclonal antibody, hypocretin agonist or antagonist, or an oligonucleotide of the present invention.

5 A therapeutically effective amount of a hypocretin polypeptide, as an example for practicing the invention, is a predetermined amount calculated to achieve the desired effect, i.e., to modulate receptor interaction with its normal target, and thereby interfere with normal receptor function. Depending on the structure of the particular peptide the binding of some peptides will activate the receptor, while binding of other peptides will not activate the receptor.

10 Similarly, a therapeutically effective amount of an anti-hypocretin antibody is a predetermined amount calculated to achieve the desired effect, i.e., to immunoreact with the hypocretin, and thereby inhibit the hypocretin receptor's ability to interact with its normal target, hypocretin, and thereby interfere with normal receptor function.

15 The in vivo inhibition of hypocretin receptor function using a hypocretin polypeptide, an anti-hypocretin antibody, or hypocretin agonist or antagonist of this invention is a particularly preferred embodiment and is desirable in a variety of clinical settings, such as where the patient is exhibiting symptoms of an over or under activated hypocretin receptor.

20 A therapeutically effective amount of a hypocretin polypeptide, agonist or antagonist of this invention is typically an amount such that when administered in a physiologically tolerable composition is sufficient to achieve a plasma concentration of from about 0.1 nanomolar (nM) to about 100 nM, and preferably from about 0.5 nM to about 10 nM.

25 A therapeutically effective amount of an antibody of this invention is typically an amount of antibody such that when administered in a physiologically tolerable composition is sufficient to achieve a plasma concentration of from about 0.1 microgram ( $\mu$ g) per milliliter (ml) to about 100  $\mu$ g/ml, preferably from about 1  $\mu$ g/ml to about 5  $\mu$ g/ml, and usually about 5  
30  $\mu$ g/ml.

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route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to  
5 maintain concentrations in the CSF or blood in the ranges specified for in vivo therapies are included.

As an aid to the administration of effective therapeutic amounts of a hypocretin polypeptide, agonist, antagonist, antibody, or monoclonal antibody, (hereinafter a "therapeutic agent") a diagnostic method of this invention for  
10 detecting a therapeutic agent in the subject's CSF or blood is useful to characterize the fate of the administered therapeutic agent. Suitable diagnostic (monitoring) assays are described herein.

b. Methods for Inhibiting Gene Expression

In another embodiment, the invention includes the use of  
15 nucleic acids encoding portions of a hypocretin gene for inhibiting gene expression and function.

The present invention provides for a method for inhibiting expression of hypocretin gene products and thereby inhibiting the function of the target hypocretin protein. The DNA segments and their compositions have a  
20 number of uses, and may be used in vitro or in vivo. In vitro, the compositions may be used to block function and expression of hypocretin in cell cultures, tissues, organs and the like materials that can express hypocretin. In vivo, the compositions may be used prophylactically or therapeutically for inhibiting expression of a hypocretin gene, and by inhibiting diseases or medical conditions  
25 associated with the expression or function of the hypocretin gene or the activity state of its receptor.

The method comprises, in one embodiment, contacting cells or tissues with a therapeutically effective amount of a pharmaceutically acceptable composition comprising a DNA segment of this invention. In a related  
30 embodiment, the contacting involves introducing the DNA segment composition

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specific uptake vehicles, and the like.

The pharmaceutical composition containing the therapeutic oligonucleotide preferably also contains physiologically acceptable carriers, in particular hydrophobic carriers which facilitate carrying the oligonucleotide through the cell membrane or blood brain barrier.

Exemplary descriptions of the delivery of therapeutic DNA segments and oligonucleotides into cells can be found in the teachings of United States Patent Nos. 5,04,820, 4,806,463, 4,757,055, and 4,689,320, which teachings are hereby incorporated by reference.

A therapeutically effective amount is a predetermined amount calculated to achieve the desired effect, i.e., to bind to a hypocretin gene present and thereby inhibit function of the gene.

As is apparent to one skilled in the art, the copy number of a hypocretin gene may vary, thereby presenting a variable amount of target with which to hybridize. Thus it is preferred that the therapeutic method achieve an intracellular concentration of a therapeutic DNA segment of this invention in molar excess to the copy number of the gene in the cell, and preferably at least a ten-fold, more preferably at least a one-hundred fold, and still more preferably at least a one thousand-fold excess of therapeutic DNA segments relative to the gene copy number per cell. A preferred effective amount is an intracellular concentration of from about 1 nanomolar (nM) to about 100 micromolar ( $\mu$ M), particularly about 50 nM to about 1  $\mu$ M.

Alternatively, a therapeutically effective amount can be expressed as an extracellular concentration. Thus it is preferred to expose a cell containing a hypocretin gene to a concentration of from about 100 nM to about 10 millimolar (mM), and preferably about 10  $\mu$ M to 1 mM. Thus, in embodiments where delivery of a therapeutic DNA segment composition is designed to expose cells to the nucleic acid for cellular uptake, it is preferred that the local concentration of the DNA segment in the area of the tissue to be treated reach the extracellular concentrations recited above.

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The active ingredient can be mixed with excipient which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipient are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

As described herein, for intracellular delivery of oligonucleotides, specialized carriers may be used which facilitate transport of the oligonucleotide across the cell membrane. These typically are hydrophobic compositions, or include additional reagents which target delivery to and into cells.

Liquid compositions can also contain liquid phases in addition to

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using the former vector (H. Usui, personal communication). The number of recombinants in the libraries were: pT7T3D hypothalamus  $8 \times 10^6$ ; cerebellum pGEM11Zf (-)  $5 \times 10^5$ ; hippocampus pGEM11Zf(-)  $1 \times 10^6$ .

Subtractive hybridization was performed in two cycles using the previously described procedure (Usui et al., *supra*). Briefly, 1  $\mu$ g of trace-labeled, tagged hypothalamus target cDNA prepared as described from the pT7T3D target library was annealed for 24 hrs at 68 degree Celsius in 10  $\mu$ l of hybridization buffer (Usui et al, *supra*) with 20  $\mu$ g cerebellum cRNA (ratio 1:20). After hydroxyapatite chromatography, the single-stranded fraction corresponded to 10% of the input material, as judged by tracer quantitation. This was mixed with 20  $\mu$ g of hippocampus cRNA (estimated ratio 1:200) for a second 24 hr hybridization, after which 30% of the input chromatographed at the single-strand position. Cumulatively, these steps removed more than 97% of the input tracer. An aliquot of the single-stranded material was used as template in a 30-cycle PCR (program: 94 degree Celsius for 15 sec, 60 degree Celsius for 15 sec, 72 degree Celsius for 1 min) using primers corresponding to the tag sequences (Usui et al., *supra*): 5'-AACTGGAAGAATTCGCGG-3' and 5'-AGGCCAAGAATTCGGCACGA-3'. The amplification product was cleaved with NotI, then EcoRI, and inserted into pBCSK<sup>+</sup>. A dot blot was prepared and screened with probes prepared from the target, subtracted target and driver libraries as previously described by Usui et al, *supra*, using serial dilutions of plasmid cDNA clones isolated previously in this laboratory. The target and subtracted target cDNA libraries were screened to determine the frequency of oxytocin and VAT-1 cDNA clones using as probes clones isolated in the present study.

Clone 35 cDNA from the subtracted rat hypothalamus library was used as a probe to screen a rat brain cDNA library in the plasmid pHG327 as described by Forss-Petter et al., *J. Mol. Neurosci.* 1:63-75 (1989). The cDNA library was constructed as described by Staeheli et al., *Cell* 44:147-158 (1986).

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hypocretin (Fig. 5). The peptide from about amino acid residue 28 to about amino acid residue 130 (SEQ ID NO:6) represents the peptide produced by cleavage of the signal peptide. The peptide from about amino acid residue 28 to about amino acid residue 66 (SEQ ID NO:7) corresponds to hcrt1. The peptide from about amino acid residue 28 to about amino acid residue 65 (SEQ ID NO:8) corresponds to hcrt1 matured by peptidylglycine alpha-amidating monooxygenase, leaving the nitrogen of the terminal glycine as a C-terminal amide in the mature peptide. The peptide from about amino acid residue 70 to about amino acid residue 97 (SEQ ID NO:9) corresponds to hcrt2. The peptide from about amino acid residue 70 to about amino acid residue 96 (SEQ ID NO:10) corresponds to hcrt2 matured by peptidylglycine alpha-amidating monooxygenase, leaving the nitrogen of the terminal glycine as a C-terminal amide in the mature peptide. The peptide from about amino acid residue 47 to about amino acid residue 66 (SEQ ID NO:11) corresponds to the consensus sequence region of hcrt1 (Fig. 5B). The peptide from about amino acid residue 78 to about amino acid residue 97 (SEQ ID NO:12) corresponds to the consensus region of Hcrt2. The peptide GNHAAGILT (SEQ ID NO:13) is common to both hcrt1 and hcrt2.

### EXAMPLE 3

Rat H35 (SEQ ID NO:3) is inserted into the BamHI sites of a pHG237 vector. Upon digestion with BamHI restriction enzyme, the resultant 569 bp fragment is then inserted directly into the BglII site of the polylinker region of the pCM 4 vector (D. Russell, U. Texas Southwestern Medical Center, Dallas, TX), which uses the cytomegalovirus (CMV) promoter. Several eight to ten amino acid epitope tags are added by PCR to the C-terminus of H35 to allow visualization of the expressed product.

The respective 5' and 3' primers, 5'

ATCGAGATCTAGACACCATGAACCTTCCTTCTACAAAGGTT 3' and 5'  
 ACTGTCTAGATCATAGATCTTCTTCAGAAATAAGTTTTGTTTCGACTCTG  
 GATCCGCCCCGGGGCGCT 3', are used as primers to amplify H35 beginning  
 at position 85 in SEQ ID NO:3 with an inserted BglII site added at its 5' end to

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Chromosome 11 that shows conserved synteny with human Chromosome 17q21-q24.

In Northern blot studies using poly(A)<sup>+</sup> RNA prepared from brain and different peripheral tissues, the 700-nucleotide hypocretin mRNA was detected only in brain samples. Previous studies with RNA from different regions of the brain had detected the hypocretin mRNA predominantly in hypothalamus samples. In samples of RNA from whole brains of developing rats, hypocretin mRNA was detected at low concentrations as early as embryonic day 18, but increased in concentration dramatically after the third postnatal week. There was no detectable difference between brain samples from adult males and females, suggesting that the late onset was not related to sexually dimorphic processes. In situ hybridization studies detected cell bodies in the dorsal-lateral hypothalamus and in cells that line the ventricles.

#### EXAMPLE 5

A polyclonal antiserum (serum 2050) was raised to a chemically synthesized peptide corresponding to the C-terminal 17 amino acid residues (CPTATATACAPRGGSRV) of the rat preprohypocretin sequence. In Western transfer blots using as target electrophoretically separated proteins from bacteria transformed with the plasmid pRSET B engineered to express preprohypocretin, a single prominent immunoreactive band was observed with a migration of approximately 19kDa with the hyperimmune serum, but not with the preimmune serum. No immunoreaction was detected with an extract from bacteria transformed with a preprohypocretin/pRSET B expression plasmid, indicating that detection of the 19kDa target requires hypocretin expression. Analogous results were obtained with an additional antiserum to the 17mer and two antisera to synthetic hcr2.

In immunohistochemical studies with antiserum 2050 on sections from perfused adult male rats, immunoreactive cell bodies were observed exclusively in the perifornical nucleus and dorsal and lateral hypothalamic areas, consistent with the *in situ* hybridization results (Fig. 4). This coincident staining,

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lateral hypothalamus in feeding behavior, blood pressure, and central regulation of immune function, although precise nuclei have not been correlated with these activities. A threshold-type response was obtained in which, at the highest dose, 10  $\mu$ g, body temperature dropped from 37.7 to 36.7 degrees Celsius over 30 minutes following administration, then recovered to normal over 2 hours. Food intake was monitored over 2 hours following administration and a 40% reduction in food intake was measured at a dose of 5 $\mu$ g. Whereas the concentrations of peptide required for an effect might seem high, they are comparable to the doses of leptin administered ICV to obtain a comparable suppression of food intake. The presumed target cells of hypocretin may not be very accessible by this unphysiological mode of administration. Local injection or intravenous administration of hypocretin might be more suitable for physiological studies.

The cell bodies that produce the hypocretins are located in an area implicated in ablation studies as regulatory centers for appetitive behaviors, suggesting that the hypocretins may serve as a major transmitters for the central system signalling the status of energy balance in the major fat repositories. The projections of hypocretin-producing cells indicate that the peptides function both within the hypothalamus and at a complex and diffuse network of targets in several regions of the brain that may coordinate the various aspects of appetitive behavior, adaptive thermogenesis and metabolic regulation.

Rat hypothalamus from 18-day embryos was cultured for 10 days in vitro. The mediobasal hypothalamus was removed from embryonic day 18 Sprague Dawley rats. The tissue was enzymatically digested in a mild protease solution (10 U/ml papain and 0.2 mg/ml L-cysteine in Earle's balanced salt solution) for 30 minutes. Next, the tissue was pelleted, and the protease solution was removed. Tissue was then suspended in standard tissue culture medium (glutamate- and glutamine-free DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 6 gm/l glucose) and then triturated into a single-cell suspension. Cells were washed and pelleted an additional three times. The single-cell suspension was plated onto 22 mm<sup>2</sup> glass coverslips that



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Table 1 Cumulative Data From 100 Clones

	Clone <sup>a</sup>	BLAST Homology <sup>b</sup>	Accession# <sup>c</sup>	# <sup>d</sup>	Pattern <sup>e</sup>
5	2 +	oxytocin	M25649	13	A/A
	6 +	VAT1-like	T05306	11	B/B
	1 +	CART	U10071	7	C
	35 +	novel		6	A/A
	15 +	novel		4	B/C
10	25 +	POMC	J00759	4	A
	12 +	novel(E)	R75926	3	B/B
	16 +	vasopressin	M25646	3	A
	18 +	glutathione perox	U13705	3	B
	29 +	novel CaM kinase		3	B/C
15	3 +	novel		2	B/C
	10 +	novel		2	B/B
	51 +	ubiquitin carrier	M91679	2	C
	62 +	novel		2	C
	5 -	calbindin	U08290		B
20	14 +	melanin-conc hormone	M62641		C
	17 +	asp aminotrans	M18467		D
	19 -	novel(E)	R74893		D
	20 +	novel			A/B
	21 -	novel(E)	T32756		A/D
25	22 -	novel			D
	33 +	novel(E)	R67552		A/A
	34 -	Cl <sup>-</sup> /HCO <sub>3</sub> <sup>-</sup> exchanger	J05167		C
	37 +	novel			B/D
	39 -	novel			C
30	45 +	novel			C
	46 +	fibromodulin	X82152		C
	47	perox enolhydratase	U08976		C
	48 +	galanin	J03624		B
	52 -	5-HT <sub>2</sub> receptor	L31546		B
35	53 +	MHC orf	M32010		E
	55 +	HNF dimer cofactor	M83740		C
	56 +	carbonyl reductase	X84349		C
	57 +	tyrosine hydroxylase	M10244		A
	63 +	novel			D
40	67 +	novel			B/B
	73 +	novel			C
	74 +	novel(E)	T93996		C
	75 +	lamin C2	D14850		A
	86 +	novel			C
45	92 -	novel(E)	R49544		C
	98 +	novel			B/D
	99 -	neuronal kinesin	U06698		B/D

<sup>a</sup> number of prototype clone in set of 100 followed by indication (+/-) as to whether 3' sequence contained poly(A)-addition hexad (no 3' sequence for clone 47)

<sup>b</sup> short name of matching species or novel for no match;(E) indicates EST match

<sup>c</sup> GenBank database reference

<sup>d</sup> number of representatives in set of 100

<sup>e</sup> hybridization pattern in cDNA library Southern assay/Northern blot assay. Code: A, target only; B, target highly enriched; C, hypothalamus and hippocampus; D, not highly enriched; E, too faint to categorize

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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

Met Asn Leu Pro Ser Thr Lys Val Pro Trp Ala Ala Val Thr Leu Leu
 1           5           10           15
Leu Leu Leu Leu Leu Pro Pro Ala Leu Leu Ser Leu Gly Val Asp Ala
          20           25           30
Gln Pro Leu Pro Asp Cys Cys Arg Gln Lys Thr Cys Ser Cys Arg Leu
          35           40           45
Tyr Glu Leu Leu His Gly Ala Gly Asn His Ala Ala Gly Ile Leu Thr
 50           55           60
Leu Gly Lys Arg Arg Pro Gly Pro Pro Gly Leu Gln Gly Arg Leu Gln
 65           70           75           80
Arg Leu Leu Gln Ala Asn Gly Asn His Ala Ala Gly Ile Leu Thr Met
          85           90           95
Gly Arg Arg Ala Gly Ala Glu Leu Glu Pro Tyr Pro Cys Pro Gly Arg
          100          105          110
Arg Cys Pro Thr Ala Thr Ala Thr Ala Leu Ala Pro Arg Gly Gly Ser
          115          120          125
Arg Val
          130

```

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 130 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Asn Phe Pro Ser Thr Lys Val Pro Trp Ala Ala Val Thr Leu Leu
 1           5           10           15
Leu Leu Leu Leu Leu Pro Pro Ala Leu Leu Ser Leu Gly Val Asp Ala
          20           25           30
Gln Pro Leu Pro Asp Cys Cys Arg Gln Lys Thr Cys Ser Cys Arg Leu
          35           40           45
Tyr Glu Leu Leu His Gly Ala Gly Asn His Ala Ala Gly Ile Leu Thr
 50           55           60

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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TAAGACGACG GCCTCAGACT TCTTGGGTAT TTGGACCACT GCACTGAAGA GATCATCTCT	60
CCAGATTACT TTCCCCTGAG CTCCAGGCAC CATGAACTTT CCTTCTACAA AGGTTCCCTG	120
GGCCGCCGTG ACGTGCTGTC TGCTGCTACT GCTGCCACCG GCGCTGCTGT CGCTTGGGGT	180
GGACGCACAG CCTCTGCCCG ACTGCTGTCG CCAGAAGACG TGTTCCCTGCC GTCTCTACGA	240
ACTGTTGCAC GGAGCTGGCA ACCACGCTGC GGGTATCCTG ACTCTGGGAA AGCGGCGGCC	300
TGGACCTCCA GGCCTCCAGG GACGGCTGCA GCGCCTCCTT CAGGCCAACG GTAACCACGC	360
AGCTGGCATC CTGACCATGG GCCGCCGCGC AGGCGCAGAG CTAGAGCCAC ATCCCTGCTC	420
TGGTCGCGGC TGTCCGACCG TAACTATCAC CGCTTTAGCA CCCCAGGGAG GGTCCGGAGT	480
TTGAACCCAT CTTCTATCCT TGTCCTGATC CAACTTCCC CCTCTGCTCG CCGCTGTCAG	540
TCTCTTGTA AATGGCAATA AAGACGTTTC TCTGTTGGTG TG	582

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1458 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTAGGAGAC ATTGCGGCGG CGGTGGCGGC GTTGGCAGCA GCTGCAGACA TGCTGCTGCT	60
CAAGAAACAG ACGGAGGACA TCAGCAGTGT CTATGAGATC CGGGAGAAGC TGGGCTCGGG	120
TGCCTTCTCT GAGGTGATGC TGGCCCAGGA AAGGGGCTCT GCTCATCTTG TGGCCCTCAA	180
GTGCATTCCC AAGAAAGCAC TTCGGGGCAA GGAGGCCCTG GTGGAGAATG AGATCGCAGT	240
ACTCCGCAGG ATTAGCCACC CCAACATTGT GGCTCTGGAG GACGTCCACG AGAGCCCTTC	300
CCATCTCTAC TTGGCCATGG AGCTGGTAAC AGGTGGTGAA CTGTTTGACC GAATCATGGA	360
GCGGGGCTCC TACACAGAGA AGGATGCGAG CCACCTTGTA GGGCAGGTCC TTGGTGCTGT	420

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Gln Gly Arg Leu Gln Arg Leu Leu Gln Ala Asn Gly Asn His Ala Ala  
 50 55 60  
 Gly Ile Leu Thr Met Gly Arg Arg Ala Gly Ala Glu Leu Glu Pro Tyr  
 65 70 75 80  
 Pro Cys Pro Gly Arg Arg Cys Pro Thr Ala Thr Ala Thr Ala Leu Ala  
 85 90 95  
 Pro Arg Gly Gly Ser Arg Val  
 100

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 39 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Gly Val Asp Ala Gln Pro Leu Pro Asp Cys Cys Arg Gln Lys Thr  
 1 5 10 15  
 Cys Ser Cys Arg Leu Tyr Glu Leu Leu His Gly Ala Gly Asn His Ala  
 20 25 30  
 Ala Gly Ile Leu Thr Leu Gly  
 35

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 38 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Gly Val Asp Ala Gln Pro Leu Pro Asp Cys Cys Arg Gln Lys Thr  
 1 5 10 15  
 Cys Ser Cys Arg Leu Tyr Glu Leu Leu His Gly Ala Gly Asn His Ala  
 20 25 30  
 Ala Gly Ile Leu Thr Leu  
 35

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(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Arg Leu Tyr Glu Leu Leu His Gly Ala Gly Asn His Ala Ala Gly Ile  
 1 5 10 15  
 Leu Thr Leu Gly  
 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 20 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Arg Leu Gln Arg Leu Leu Gln Ala Asn Gly Asn His Ala Ala Gly Ile  
 1 5 10 15  
 Leu Thr Met Gly  
 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 9 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Asn His Ala Ala Gly Ile Leu Thr  
 1 5

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sequence of (SEQ ID NO:7);

(b) a polynucleotide encoding a polypeptide comprising the sequence of (SEQ ID NO:8);

5 (c) a polynucleotide encoding a polypeptide comprising amino acids 42 to 66 of (SEQ ID NO:1);

(d) a polynucleotide encoding a polypeptide comprising amino acids 42 to 65 of (SEQ ID NO:1);

(e) a polynucleotide encoding a polypeptide comprising amino acids 43 to 66 of (SEQ ID NO:1);

10 (f) a polynucleotide encoding a polypeptide comprising amino acids 43 to 65 of (SEQ ID NO:1); and

(g) a polynucleotide capable of hybridizing to and which is at least 95% homologous to a polynucleotide of (a) through (f).

15 6. An isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide encoding a polypeptide comprising the sequence of (SEQ ID NO:9);

(b) a polynucleotide encoding a polypeptide comprising the sequence of (SEQ ID NO:10); and

20 (c) a polynucleotide capable of hybridizing to and which is at least 95% homologous to the polynucleotide of (a) or (b).

7. An isolated polynucleotide selected from the group consisting of:

25 (a) a polynucleotide encoding a polypeptide comprising amino acids 100 to 130 of (SEQ ID NO:1);

(b) a polynucleotide encoding a polypeptide comprising amino acids 100 to 130 of (SEQ ID NO:2);

(c) a polynucleotide encoding a polypeptide comprising amino acids 100 to 111 of (SEQ ID NO:1);

30 (d) a polynucleotide encoding a polypeptide comprising amino

(h) the amides thereof.

12. An isolated polypeptide selected from the group consisting of:

- 5 (a) a polypeptide comprising the sequence of SEQ ID NO:9;  
(b) a polypeptide comprising the sequence of SEQ ID NO:10;  
and

(c) the amides thereof.

13. An isolated polypeptide selected from the group consisting of:

10 (a) a polypeptide comprising amino acids 100 to 130 of (SEQ ID NO:1);

(b) a polypeptide comprising amino acids 100 to 130 of (SEQ ID NO:2);

(c) a polypeptide comprising amino acids 100 to 111 of (SEQ ID NO:1);

15 (d) a polypeptide comprising amino acids 100 to 110 of (SEQ ID NO:1);

(e) a polypeptide comprising amino acids 114 to 130 of (SEQ ID NO:1);

20 (f) a polypeptide comprising at least one conservative amino acid substitution in the sequence of polypeptides (a - e); and

(g) the amides thereof.

14. A vector comprising a polynucleotide of claim 1 operably linked to control sequences which direct the expression of the polynucleotide.

25 15. A vector comprising a polynucleotide of claim 2 operably linked to control sequences which direct the expression of the polynucleotide.

16. A vector comprising a polynucleotide of claim 3 operably linked to control sequences which direct the expression of the polynucleotide.

30 17. A vector comprising a polynucleotide of claim 4 operably linked to control sequences which direct the expression of the polynucleotide.

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H35 protein.

36. An antibody of claim 35 which is a monoclonal antibody.

37. A kit for detecting the presence of an H35 protein in a mammalian sample comprising an antibody which immunoreacts with a mammalian H35 protein or with a polypeptide of claim 10 in an amount sufficient for at least one assay and suitable packaging material.

38. A kit for detecting the presence of an H35 protein in a mammalian sample comprising an antibody which immunoreacts with a mammalian H35 protein or with a polypeptide of claim 11 in an amount sufficient for at least one assay and suitable packaging material.

39. A kit for detecting the presence of an H35 protein in a mammalian sample comprising an antibody which immunoreacts with a mammalian H35 protein or with a polypeptide of claim 12 in an amount sufficient for at least one assay and suitable packaging material.

40. A kit for detecting the presence of an H35 protein in a mammalian sample comprising an antibody which immunoreacts with a mammalian H35 protein or with a polypeptide of claim 13 in an amount sufficient for at least one assay and suitable packaging material.

41. The kit of claim 37 further comprising a detecting antibody which binds to the anti-H35 antibody.

42. The kit of claim 41 wherein the detecting antibody is labeled.

43. The kit of claim 42 wherein the label comprises enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, phosphorescent compounds, or bioluminescent compounds.

44. A kit for detecting the presence of genes encoding an H35 protein comprising a polynucleotide of claim 1, or fragment thereof having at least 10 contiguous bases, in an amount sufficient for at least one assay, and suitable packaging material.

45. A method for detecting the presence of a nucleic acid



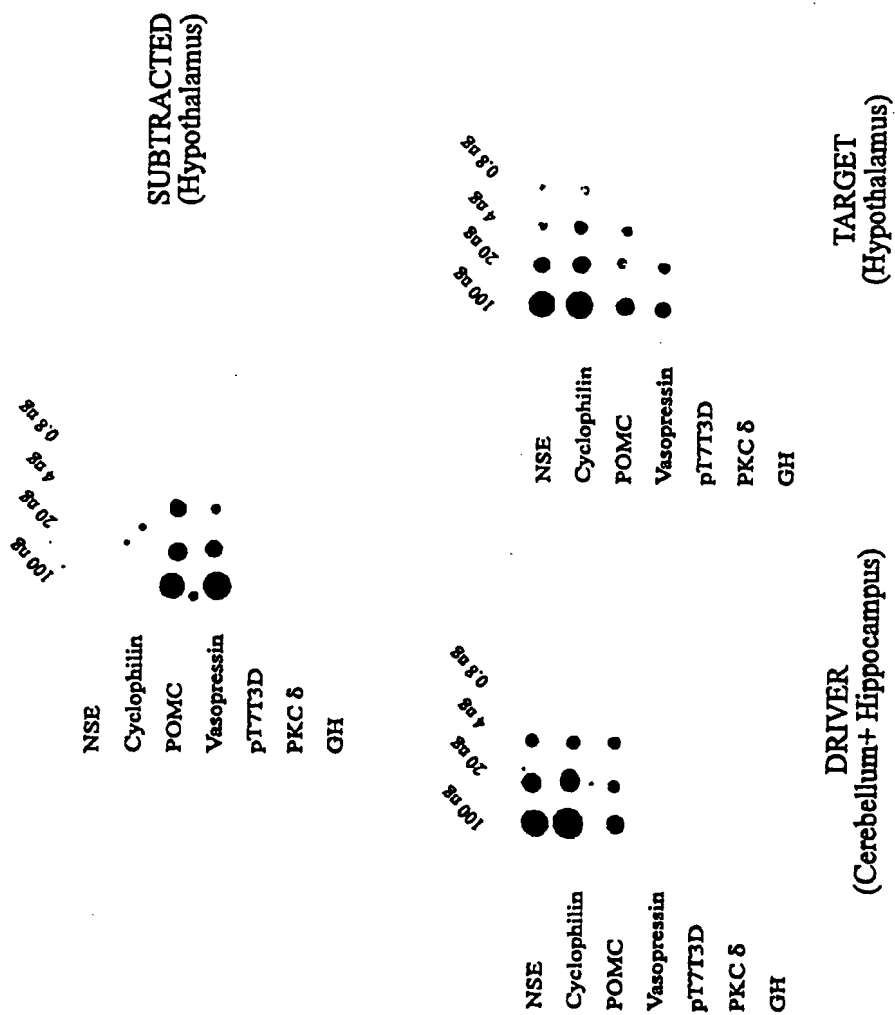


Fig. 1

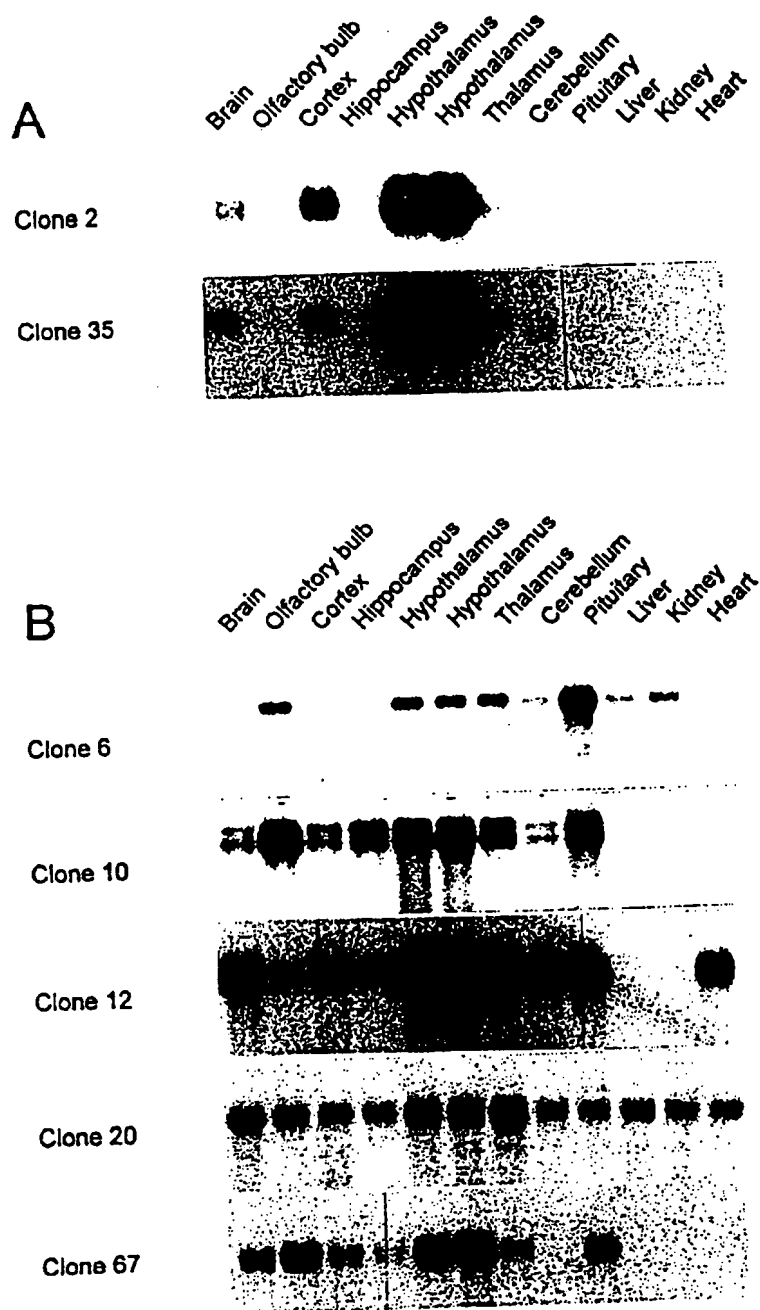
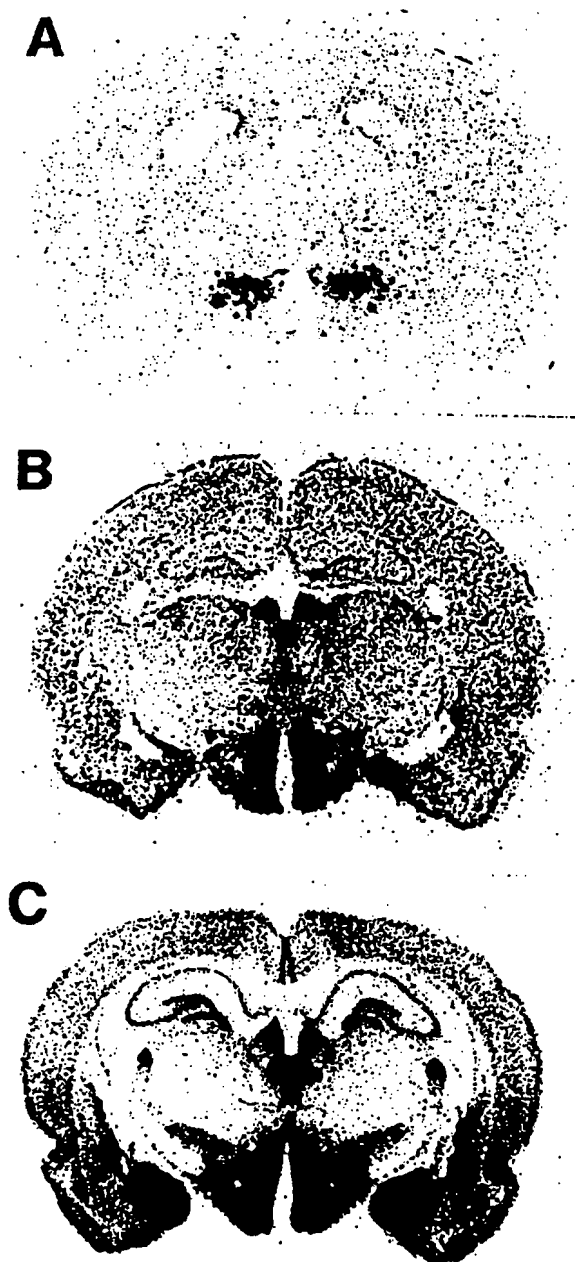


Fig. 3



**Fig. 4**

## A

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M  N  L/F P  S  T  K  V  P  W  A  A  V  T  L  L
ATG AAC CTT CCT TCT ACA AAG GTT CCC TGG GCC GCC GTG ACG CTG CTG
ATG AAC TTT CCT TCT ACA AAG GTT CCC TGG GCC GCC GTG ACG CTG CTG
*
L  L  L  L  L  P  P  A  L  L  S  L  G  V  D  A
CTG CTG CTA CTG CTG CCG CCG GCG CTG CTG TCG CTT GGG GTG GAC GCG
CTG CTG CTA CTG CTG CCG CCG GCG CTG CTG TCG CTT GGG GTG GAC GCA
*
Q  P  L  P  D  C  C  R  Q  K  T  C  S  C  R  L
CAG CCT CTG CCC GAC TGC TGT CGC CAG AAG ACG TGT TCC TGC CGT CTC
CAG CCT CTG CCC GAC TGC TGT CGC CAG AAG ACG TGT TCC TGC CGT CTC
*
Y  E  L  L  H  G  A  G  N  H  A  A  G  I  L  T
TAC GAA CTG TTG CAC GGA GCT GGC AAC CAC GCC GCG GGC ATC CTC ACT
TAC GAA CTG TTG CAC GGA GCT GGC AAC CAC GCT GCG GGT ATC CTG ACT
*
L  G  K  R  R  P  G  P  P  G  L  Q  G  R  L  Q
CTG GGA AAG CGG CGA CCT GGA CCC CCA GGC CTC CAA GGA CGG CTG CAG
CTG GGA AAG CGG CGG CCT GGA CCT CCA GGC CTC CAG GGA CGG CTG CAG
*
R  L  L  Q  A  N  G  N  H  A  A  G  I  L  T  M
CGC CTC CTT CAG GCC AAC GGT AAC CAC GCA GCT GGC ATC CTG ACC ATG
CGC CTC CTT CAG GCC AAC GGT AAC CAC GCA GCT GGC ATC CTG ACC ATG
*
G  R  R  A  G  A  E  L  E  P  Y/H P  C  P/S G  R
GGC CGC CGC GCA GGC GCA GAG CTA GAG CCA TAT CCC TGC CCT GGT CGC
GGC CGC CGC GCA GGC GCA GAG CTA GAG CCA CAT CCC TGC TCT GGT CGC
*
R/G C  P  T  A/V T  A/T T  A  L  A  P  R  G  G  S
CGC TGT CCG ACT GCA ACC GCC ACC GCT TTA GCG CCC CGG GGC GGA TCC
GGC TGT CCG ACC GTA ACT ACC ACC GCT TTA GCA CCC CGG GGA GGG TCC
*
R/G V
AGA GTC TGA
GGA GTC TGA
*

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## B

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consensus:          RL LL  GNHAAGILT G
hcert1:    LGVDAQPLPDCCRQKTCSCRLYELLHGAGNHAAGILTLG
hcert2:          PGPPGLQGRLQRLQLQANGNHAAGILTMG
SECRETIN:    HSDGTFTSKLSRLRDSARLQRLQLGLV HSDGTFTSK
              * * * * *

```

Fig. 5

541 AGCTGGCAACATGCTAGGCACAGCCTGTGGGACCCAGGATATGTGGCCCCAGAGCTCCT 500  
-----+-----+-----+-----+-----+  
TCGACCGTTGTACGATCCGTGTTCGGACACCCCTGGGGTCTTATACACCGGGGTCTCGAGGA  
A G N M L G T A C G T P G Y V A P E L L

601 GGAGCAGAAACCTACGGGAAGGCCGTAGATGTGTGGGCCCTGGGTGTCTCTCTACAT 660  
-----+-----+-----+-----+-----+  
CCTCGTCTTTGGGATGCCCTTCCGGCATCTACACACCCGGGACCCACAGTAGAGGATGTA  
E Q K P Y G K A V D V W A L G V I S Y I

661 CCTGCTGTGTGGGTACCCCCCTTCTATGATGAGAGCGATCCTGAACTCTTCAGCCAGAT 720  
-----+-----+-----+-----+-----+  
GGACGACACACCCATGGGGGGGAAGATACTACTCTCGCTAGGACTTGAGAAGTCGGTCTA  
L L C G Y P P F Y D E S D P E L F S Q I

721 TCTGAGGGCCAGCTACGAGTTTGACTCTCCCTTTTGGGATGACATCTCAGAATCAGCCAA 780  
-----+-----+-----+-----+-----+  
AGACTCCCGGTCGATGCTCAAACCTGAGAGGGGAAACCCCTACTGTAGAGTCTTAGTCGGTT  
L R A S Y E F D S P F W D D I S E S A K

781 AGACTTCATTGGGCACCTTCTGGAACGTGATCCCCAGAAGAGGTTACCTGCCAACAGGC 840  
-----+-----+-----+-----+-----+  
TCTGAAGTAAGCCGTGGAAGACCTTGCACTAGGGGTCTTCTCCAAGTGGACGGTTGTCCG  
D F I R H L L E R D P Q K R F T C Q Q A

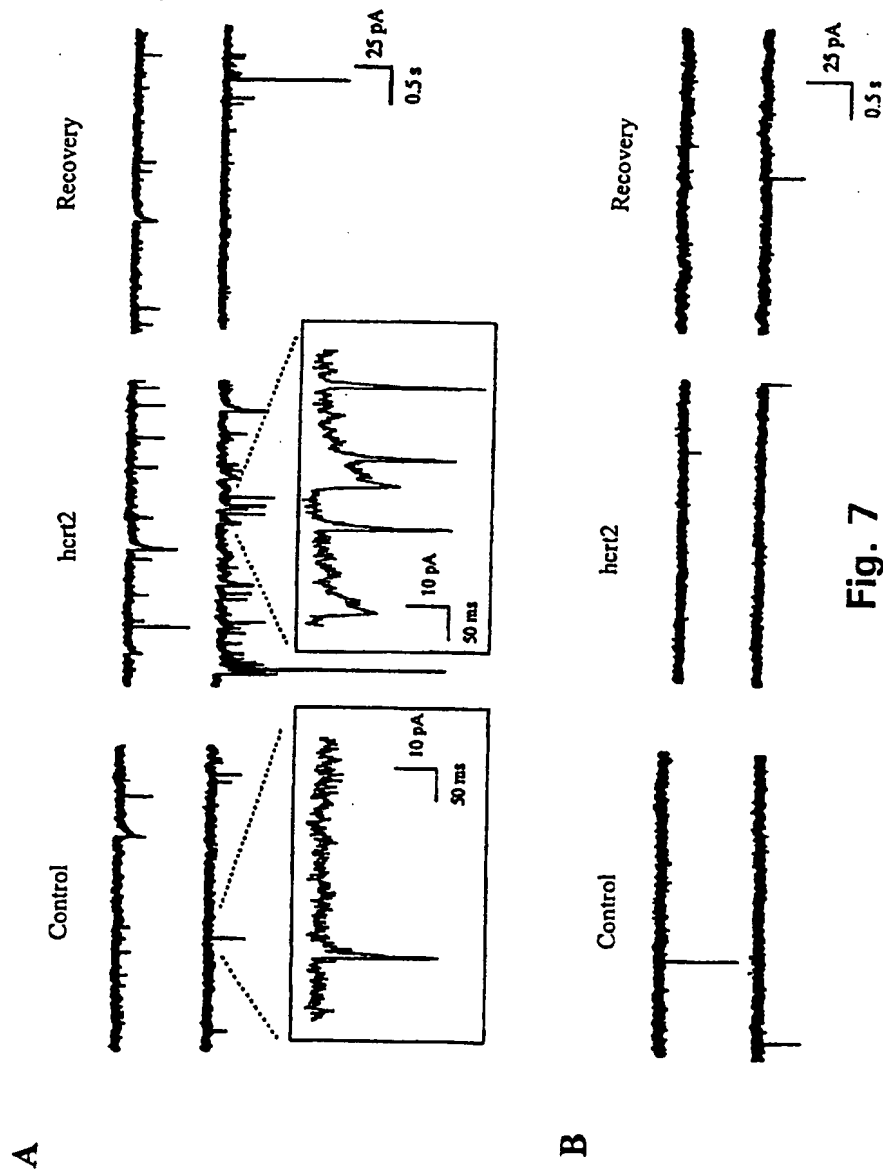
841 CTTACAGCATCTCTGGATCTCTGGGGATGCAGCCTTGGACAGGGACATCCTAGGTTCTGT 900  
-----+-----+-----+-----+-----+  
GAATGTCGTAGAGACCTAGAGACCCCTACGTCGGAACCTGTCCCTGTAGGATCCAAGACA  
L Q H L W I S G D A A L D R D I L G S V

901 CAGTGAGCAGATCCAGAAGAATTTTCCAGGACCCACTGGAAGCGTGCAATCAATGCCAC 960  
-----+-----+-----+-----+-----+  
GTCACCTCGTCTAGGTCTTCTTAAACGGTCTCTGGGTGACCTTCGCACGTAAGTTACGGTG  
S E Q I Q . K N F A R T H W K R A F N A T

961 ATCATTCTACGTCACATCCGTAAGCTGGGACAGAGCCAGAGGGTGAGGAAGCCTCCAG 1020  
-----+-----+-----+-----+-----+  
TAGTAAGGATGCAGTGTAGGCATTGACCCCTGTCTCGGGTCTCCCACTCCTCCGGAGGTC  
S F L R H I R K L G Q S P E G E E A S R

1021 GCAGGGTATGACCCGTCACAGCCACCCAGGCCTTGGGACTAGCCAGTCTCCCAAGTGGTG 1080  
-----+-----+-----+-----+-----+  
CGTCCCATACTGGGCAGTGTCTGGTGGGTCCGGAACCCCTGATCGGTGAGAGGGTTACCCAC  
Q G M T R H S H P G L G T S Q S P K W V

Fig. 6 (cont.)



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/13657

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHARNOCK-JONES et al. Extension of incomplete cDNAs (ESTs) by biotin/streptavidin-mediated walking using the polymerase chain reaction. Journal of Biotechnology. 30 June 1994, Vol. 35, No. (2-3), pages 205-215.	55-57
Y	HUDSON T. Human STS WT-10041. GENBANK Accession No. G11664. 19 October 1995, see entire document.	44
A	FEHMANN et al. Cell and molecular biology of the incretin hormones glucagon-like peptide-I and glucose-dependent insulin releasing polypeptide. Endocrine Reviews. 1995, Vol. 16, No. 3, pages 390-410.	1-12(1), 12(2)-13(1), 13(2)-54

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/13657

A. CLASSIFICATION OF SUBJECT MATTER:  
IPC (6):

A61K 38/04, 38/08, 38/22, 48/00; G01N 33/53, 33/567; C12Q 1/68; C12N 1/15, 1/21, 9/12, 15/09, 5/10, 15/16, 15/54, 15/63; C07K 16/26

A. CLASSIFICATION OF SUBJECT MATTER:  
US CL :

435/6, 7.1, 7.21, 7.24, 7.9, 7.92, 194, 252.3, 254.11, 320.1, 325; 514/12, 13, 15, 44; 530/309, 324, 326, 328, 387.9, 388.24, 389.2, 391.1, 399; 536/23.51, 24.31

B. FIELDS SEARCHED

Minimum documentation searched  
Classification System: U.S.

435/6, 7.1, 7.21, 7.24, 7.9, 7.92, 194, 252.3, 254.11, 320.1, 325; 514/12, 13, 15, 44; 530/309, 324, 326, 328, 387.9, 388.24, 389.2, 391.1, 399; 536/23.51, 24.31

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

GENBANK, EMBL, OENESEQ, EST-STS, SWISPROT, PRJR, PIR51, ISSUED, USPATFUL, MEDLINE, BIOSIS, CAPLUS, WPIDS, SCISEARCH, EMBASE

search terms: SEQ ID NOS:1-5, hypocretin, hypothalamus, homeostatic

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-12(1), 12(2)-13(1), 13(2)-54, drawn to polynucleotides, the encoded polypeptides, vectors and host cells comprising the polynucleotides, antibodies against the protein and kits comprising the antibodies, methods of using the antibodies for the detection of the protein, pharmaceutical compositions comprising the polypeptide, a method of using the polynucleotide for gene therapy, a method of using the polynucleotides to detect nucleic acids encoding the proteins.

Group II, claim(s) 55-57, drawn to polynucleotides encoding a second protein, and vectors and host cells comprising the polynucleotide.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the groups are drawn to polynucleotides with no structural relationship, because each polynucleotide encodes a structurally dissimilar protein having different functions.